On the Optical Rotatory Power of Crystalline Ovalbumin and Serum Albumin.

By Elrid Gordon Young, Ramsay Memorial Fellow.

(Communicated by Prof. F. G. Hopkins, F.R.S.—Received July 6, 1921.)

(From the Biochemical Laboratory, Cambridge University.)

The need for a physical method by means of which it would be possible to recognise a chemical individual of the protein group of compounds has undoubtedly been one of the factors contributing to the difficulty of research into the chemistry of the proteins. The present methods of chemical analysis do not nearly approach sufficient accuracy to distinguish between successive recrystallisations of a protein substance. The first serious effort to prove that an individual protein could be isolated is to be found in the publication of Hopkins (1900). It was here shown that a protein could be prepared with a constant specific rotation for successive recrystallisations and for material obtained from different sources. Unfortunately, this desirable physical constant, which is independent of the degree of colloidal dispersion, has been shown to vary with variations in physical and chemical conditions. Thus Alexander (1896) found the specific rotation of certain globulins varied according to the concentration of the protein and of salt present. An investigation of this phenomenon by Pauli, Samec and Strausz (1914) confirmed the observations of Hopkins (1900) and of Osborne (1899) that the presence of neutral salts has no influence on the optical rotation of a protein. The addition of acids and of alkalies they found to increase the rotation of polarised light, while the degree of change depended upon the nature of the anion in the case of an acid and of the kation in the case of a base. These observations were made, unfortunately, with the mixture of proteins contained in ox or horse serum which had simply been dialysed until salt-free. The nature of these changes may find explanation in a tautomeric equilibrium of the lactam-lactim type in the protein main chain when in aqueous solution, as suggested by Robertson (1912) and Sörensen (1912), and to which view Pauli inclines.

$$\underset{\text{Lactam formula.}}{\mathrm{R{-}CO{-}NH{-}R}} \rightleftarrows \underset{\text{Lactim formula.}}{\mathrm{R{-}C(OH)=N{-}R.}}$$

The determination of the specific rotation of crystalline ovalbumin as a means of discovering its purity, as Hopkins had shown possible, was called into question by Willcock (1908), on the basis of the test used to show when the analysis sample had been washed free from sulphate. Using a ring test with

$BaCl_2$, Willcock claims that a very much greater sensitivity can be obtained; and by employing this method, specific rotations of different albumin crystallisations were found to vary considerably. If that fact were true, the biological chemist would still be without a means of recognising a chemical individual of the protein group, even if he had isolated one.

The experimental history as described in the following pages was developed with the object of discovering whether a constant specific rotation could be obtained for crystalline ovalbumin, and what was the relation between the hydrogen ion concentration and the optical rotation with reference to the iso-electric point. It was thought that such a study might yield an explanation of the variability of the rotatory power and possibly some direct evidence on the question of a tautomeric equilibrium in the protein molecule. These experiments are described first. The investigation was extended to a study of the conditions governing the preparation of a pure crystalline serum albumin and the properties of this substance examined.

EXPERIMENTAL.

The Specific Rotation of Crystalline Ovalbumin.

The values obtained for the specific rotation of hen ovalbumin by different observers at different times and with material under somewhat different physical conditions have been recorded in Table I. The earlier determinations on amorphous material are interesting in that they show a higher value than that observed for crystalline material. Osborne and Campbell (1900) have shown that the non-crystallisable albumin, which they have called conalbumin, has a higher value for $[\alpha]_D$ than the crystalline, and contamination by this substance probably explains the higher value.

Table I.

Observer.	Material.	Specific rotation.
Haas (1876)	Amorphous	−38°·08
Panormoff (1898)	"	−36·2
Panormoff (1898)	Crystalline	−23·6
Worms (1898)	"	−26·1
Bondzynski and Zoja (1894)	"	−26·0 to −42·54
Osborne (1899)	"	−28·42
Osborne and Campbell (1900)	"	−28·60 to −30·80
Hopkins (1900)	"	−30·7
Willcock (1908)	"	−30·3 to −31·6

Only the last three determinations are of importance, as they were made on similar material which had been recrystallised several times, and of these

Hopkins alone obtained a constant value for successive recrystallisations. The earlier figures were obtained from material crystallised but once, and by the inferior technique of Hofmeister in most cases.

The last worker on the subject claims that the true value is higher than $-30{\cdot}7^\circ$ and attributes her average result of $-31{\cdot}0^\circ$ as due to a more sensitive test for the presence of sulphate ions, thus allowing of more thorough washing of the coagulated albumin before drying and weighing. The technique which I have adopted in obtaining the following observations involved the use of a 400-mm. polarimeter tube, so that very large rotations were observed. A large Hilger polarimeter was employed and observations were made both with the sodium flame and with the green band of the quartz mercury-vapour lamp. The solutions used were made as concentrated as possible, being about 10 per cent. strength. The analysis of these solutions for their content of albumin was carried out in the first series by the Devoto method, using accurately calibrated pipettes for volume measurement of a quantity of solution sufficient to yield about 0·5 grm. of dried coagulum, and Kahlbaum's purest $(NH_4)_2SO_4$ in saturated solution as coagulating agent. This solution is naturally acid, having a P_H of 5·5 or less. Coagulation was brought about by placing the solution to which the albumin had been added for 1 hour on a boiling water bath. After cooling, the precipitate was filtered off on to hardened filter paper and washed with cold water until free from sulphate. Finally, the precipitate was removed quantitatively to a platinum basin by means of a few cubic centimetres of distilled water, the excess water was evaporated on a water bath, and the protein dried until of constant weight in a Lothar Meyer air bath at 110° C.

The removal of the last traces of sulphate from the precipitate is a difficult operation. I have tested the sensitivity of the $BaCl_2$ test for sulphates, applied as a ring and in the direct way, on a series of dilutions of normal H_2SO_4. When carried out as a ring test, using a saturated solution of $BaCl_2$ and allowing the tubes to stand 15 minutes before decision, the test is sensitive to one part in one million. The same limit was found for the test when applied by adding five drops to about 20 c.c. of filtrate and allowing to stand 15 minutes. An approximate idea of the quantity of sulphate being removed can be obtained in the final stage of washing by comparison of the turbidity obtained with that given by known strengths of H_2SO_4. By continuous washing, I have never been able to remove the last traces of sulphate in less than 4 days. This point is discussed below. If any protein goes back into solution during this long period of washing, the amount must be negligible, for I have repeatedly tested the filtrates for protein with negative results, and evaporated down quantities of the filtrate to dryness without obtaining any visible residue. Table II gives the results of a series of crystallisations:—

Table II.

Crystallisation.	Second.	Third.	Fourth.	Fifth.	Sixth.	Average.
Concentration (per cent.)	7·31	5·43	11·17	9·25	11·09	
P_H	5·3	4·9	4·9	5·1	4·9	
α_D	−8·87°	−6·70°	−13·80°	−11·29°	−13·72°	
α_E	−10·80	−8·16	−16·73	−13·77	−16·73	
$[\alpha]_D$	−30·34	−30·82	−30·87	−30·61	−30·89	−30·81°
$[\alpha]_E$	−36·93	−37·36	−37·74	−37·34	−37·63	−37·53

The values obtained for the specific rotation of successive recrystallisations is thus in very good agreement with that observed by Hopkins previously with similar material.

On account of the difficulty of washing free from sulphate encountered in the Devoto method, I have considered it of interest to compare this procedure with the simpler one of coagulating the sample for analysis in a buffer mixture at the isoelectric point. The most convenient buffer mixture for this purpose is a solution of acetic acid and sodium acetate in equal molecular proportions. Thus, 5 c.c. of a N/1 CH_3COOH solution were mixed with 5 c.c. of a N/1 CH_3COONa solution, and the whole diluted to 50 c.c. This solution has a P_H of 4·74. To this mixture was added the volume of albumin solution, containing about half a gramme of protein (5 to 10 c.c.). The containing vessel was then heated for half an hour on a boiling water bath, to coagulate the albumin completely. It was then allowed to cool, filtered off on to hardened filter paper, and washed until free from sulphate. The washing period is much briefer than when $(NH_4)_2SO_4$ is used as coagulating medium. But, even with this procedure, sulphate ions are very slowly removed after the first day's washing at the rate of about 1 mgrm. per 100 c.c. of filtrate.

Table III represents the specific rotatory power observed in a series of crystallisations by the use of both methods of coagulation simultaneously carried out, and it also shows a beautiful agreement between the determinations of the albumin concentration by the two methods used. The specific rotations obtained are particularly interesting, in that they are constant in magnitude after the second crystallisation, yet slightly lower than the series reported in Table II. This suggests two possibilities. There might be slight molecular differences in the albumin of two different lots of eggs hitherto undetected by our crude methods of analysis, or, what is much more probable, the crystals obtained in the first crystallisation might be a slightly different

Table III.

Crystallisation.	Second.	Third.	Fourth.	Fifth.	Average.
Concentration (per cent.)—					
(1) $(NH_4)_2SO_4$ method ...	9·87	10·95	15·43	12·64	
(2) Buffer method	9·89	10·96	15·42	12·66	
P_H	5·4	5·4	5·3	5·3	
α_D	−11·96°	−13·22°	−18·52°	−15·22°	
α_E	−14·66	−16·12	−22·58	−18·57	
$[\alpha]_D$	−30·26	−30·17	−30·02	−30·10	−30·14°
$[\alpha]_E$	−37·09	−36·79	−36·61	−36·70	−36·80

salt from that obtained previously. This possibility, more fully discussed in the notes on the method of preparation, suggested the desirability of studying the variation of the specific rotation in relation to the hydrogen ion concentration of the solution.

The Influence of P_H *on Rotatory Power.*

The condition in which the protein molecule is isoelectric to the electric current has such a profound influence upon its physical properties that it was thought that a study of the relation of this condition to the optical rotation might yield an explanation of the divergent values recorded in the literature. For the purpose of experimentation, crystalline material, which had been crystallised four to six times, was used. The last precipitate of crystals was centrifuged at high speed from its mother liquor and redissolved in distilled water. The resulting solution thus contained a small amount of $(NH_4)_2SO_4$. The changes in hydrogen ion concentration of the solution were followed by means of various indicators, using the standard buffer mixtures devised by Clark and Lubs (1917). A series of reagents was prepared by the use of material which had been purified to the standard required and dissolved in water which had been twice distilled in glass, firstly from $KMnO_4$ and H_2SO_4, and secondly from $Ba(OH)_2$. All test-tubes employed were of uniform diameter, of clear, colourless glass, and had been steeped in dichromate cleaning mixture for 24 hours, and then steamed in superheated steam for 1 hour.

After some experimentation, the following series of indicators was selected as most suitable for use in solutions containing both protein and salt:—

P_H range.	Indicator.
1·2— 2·8	Thymol blue.
4·4— 6·0	Methyl red.
6·4— 7·8	Neutral red.
7·8— 8·4	Phenol red.
8·4—10·0	Phenolphthalein.

To eliminate the possible errors due to the presence of proteins and salts, the method of dilution was adopted. Thus, 1 c.c. of fluid was pipetted into a test-tube, 4 c.c. of water (CO_2-free) added, and a suitable quantity of the indicator. A comparator was employed in the case of any turbidity or foreign coloration. This method of dilution was later verified by the use of the hydrogen electrode and potentiometer, and found to give correct values. The values obtained with the above indicators were also verified by the electrical method on test solutions. Methyl red, neutral red and phenol phthalein give very trustworthy figures.

Experiment 1.—A concentrated solution was quickly prepared from freshly crystallised material, filtered and placed in a 400-mm. tube. Its rotation was observed at intervals in a polarimeter for several days and found to remain absolutely constant from the first observation of $-15{\cdot}35°$. The P_H of this solution was found to be 4·9. To this solution was added normal H_2SO_4 until the acidity was increased to P_H 3·75. Within a few minutes a very finely divided precipitate began to form which remained suspended and could be neither filtered nor centrifuged off. Further addition of acid so that the P_H became 2·7 merely increased the fine insoluble precipitate. A slow denaturation was apparently taking place and the solution was extremely sensitive to mechanical shock, immediately forming films of denatured material.

Experiment 2.—A fresh solution of P_H 4·9 was made alkaline by the addition of several drops of concentrated NaOH solution and the variation in the degree of rotation observed in a 200-mm. tube is recorded in Table IV:—

Table IV.

Date.	Temperature.	P_H.	α_E.
	° C.		°
December 10	11·0	4·9	−1·91
„ 13	11·0	4·9	−1·91
„ 15	11·0	4·9	−1·91
„ 15	11·0	6·65	−1·72
„ 16	11·5	6·65	−1·73
„ 19	12·0	8·4	−1·78
„ 20	12·0	8·4	−1·79
„ 21	12·0	8·4	−1·80

A distinct though small drop in rotatory power was observed and on further increasing the hydroxyl ion concentration the value for the rotation increased slowly.

The effect of adding concentrated NH_4OH to a fresh solution of albumin was next tried, employing a higher concentration of protein. Precisely the same phenomenon of a primary drop in rotatory power followed by a slow rise was observed as recorded in Table V:—

Table V.

Time interval.	Temperature.	P_H.	α_E.
	° C.		°
—	15·0	4·85	−4·25
24 hours	16·0	4·85	−4·26
10 minutes	15·0	9·15	−3·94
3 hours	15·0	9·15	−3·94
1 day	15·0	9·15	−3·95
2 days	15·0	9·15	−3·96
5 days	15·0	9·15	−3·99

Experiment 3.—The effect of both acid and alkali was demonstrated by means of the addition of very small quantities of HCl and NH_4OH and the rotations observed are recorded in Table VI:—

Table VI.

Time interval.	Temperature.	P_H.	α_D.	$[\alpha]_D$.
	° C.		°	°
—	11	5·3	−4·22	−31·89
5 minutes	11	7·5	−4·07	−30·76
30 minutes	11	7·5	−4·08	−30·83
2 hours	11	7·5	−4·08	−30·83
5 minutes	12	7·8	−4·10	−31·00
1 day	12	7·8	−4·22	−31·89
5 minutes	12	4·8	−4·40	−33·25
1 hour	12	4·8	−4·40	−33·25
2 days	13	4·8	−4·41	−33·33

The effect of the addition of alkali is most interesting and the prompt fall in the rotation followed by a slow rise to the original value is distinctly suggestive of a tautomeric equilibrium. I have observed this same phenomenon in the case of denatured serum albumin. The effect of increasing the hydrogen ion concentration of the solution is an instantaneous increase in the rotatory power, which remains constant at the new level.

The experiment was repeated in order to discover at what P_H the change in the rotatory power occurred, and to discover further if the change were reversible from either side of the isoelectric point. The observations are recorded in Table VII:—

Table VII.

Date.	P_H.	α_E.	$[\alpha]_E$.	Date.	P_H.	α_E.	$[\alpha]_E$.
		°	°			°	°
April 20 ...	4·9	−8·37	−37·79	April 20 ...	4·9	−8·37	−37·79
„ 20 ...	4·65	−8·53	−38·48	„ 20 ...	5·4	−8·09	−36·50
„ 21 ...	4·65	−8·58	−38·72	„ 21 ...	5·4	−8·10	−36·55
„ 22 ...	4·65	−8·63	−38·94	„ 22 ...	5·4	−8·11	−36·59
„ 23 ...	4·3	−8·74	−39·43	„ 23 ...	7·6	−8·14	−36·73
„ 26 ...	4·3	−8·72	−39·34	„ 23 ...	7·6	−8·15	−36·78
„ 30 ...	4·3	−8·73	−39·39	„ 25 ...	7·6	−8·14	−36·73
„ 30 ...	6·45	−8·06	−36·37	„ 26 ...	7·6	−8·20	−37·00
May 2 ...	6·45	−8·05	−36·32	„ 29 ...	7·6	−8·25	−37·23
„ 4 ...	7·1	−8·61	−38·85	„ 30 ...	7·6	−8·25	−37·23
„ 5 ...	7·1	−8·67	−39·12				

Note on the Crystallisation of Ovalbumin.

The *rôle* of acid in the process of crystallisation of albumin has received some attention at the hands of a number of investigators since the procedure was first carried out by Hopkins and Pinkus. These investigators found that a 10 per cent. solution of acetic acid served admirably for the adjustment of the solution so that crystals were deposited. Osborne recommended HCl very shortly afterwards, while Krieger found H_2SO_4 to give the best results. But as Hopkins pointed out in 1900 almost any moderately strong acid will bring about crystallisation. In his extensive study of the emulsoid colloid using crystalline ovalbumin as material, Sörensen (1917) employed N/5 H_2SO_4 with additional water and $(NH_4)_2SO_4$ solution.

In the course of my experience I have had to prepare many small lots of crystals and have employed several different acids of various strengths but always adjusting the reaction finally to about the same optimum hydrogen ion concentration. On several occasions I have found the method of Hopkins to fail to yield crystals at the beginning, although the two possible causes of failure which he mentions were excluded, viz., staleness of the eggs and insufficient whipping. If, after a heavy amorphous precipitate has been thrown down while standing after the usual adjustment, more acid be added, crystals sometimes appear on further standing. These failures I attribute to an increased alkalinity in the egg whites and inability to attain the proper hydrogen ion concentration for crystallisation before the protein was precipitated.

On two occasions when the optimum conditions had been established for crystallisation and a generous sowing of crystals added, a heavy precipitate was obtained, which consisted entirely of round globules of various sizes—the globuliths of the Hofmeister method of preparation. These globuliths could never be induced to crystallise, in spite of sowing and repeated agitation. They remained for two months in this condition, slowly coalescing into a hard gel formation at the bottom of the beaker. They were then redissolved and reprecipitated by $(NH_4)_2SO_4$, but again the precipitate consisted of nothing but globuliths of an enormous size, which again settled to a gel. This observation is of importance in the light of two recently published papers. Bradford (1920) claims to have crystallised gelatin, and Oswald (1915) albumin from human ascitic fluid, yet in both cases only globuliths were obtained. In Table VIII I have collected the amount of acetic acid used in crystallisation of several lots of egg white and expressed it in terms of cubic centimetres per 100 c.c. of filtrate:—

Table VIII.

Lot.	Fluid volume.	P_H.	Acid volume.	Product.
	c.c.		c.c.	
1	500	4·9	3·32	Crystals.
2	275	5·1	2·94	Crystals.
3	600	4·8	3·70	Globuliths.
4	650	5·0	8·56	Globuliths.
5	325	4·9	1·88	Crystals.
6	325	4·7	2·95	Crystals.

It is thus shown that the globulith mother liquors required more acid to adjust them to the conditions of precipitation than the normal. Now it has been observed by both Hopkins and Sörensen that this is necessary for eggs that are not strictly fresh. The phenomenon thus suggests to my mind further evidence toward an explanation of the *rôle* of acid and sulphate in crystallisation. We are dealing essentially with an equilibrium between albumin, water, salt and acid. The function of the salt is one of dehydration, as has been shown by Chick and Martin (1913). The function of the acid is probably twofold. By virtue of the fact that albumin is an ampholyte and on the alkaline side of its isoelectric point, it is bound to form a salt with free acid. At the same time, the acid ions have dehydrating powers. It is thus an adjustment of the available water molecules between the protein or protein salt, on the one hand, and the ammonium sulphate with the free acid ions, on the other. Sörensen (1917) has brought forward evidence from careful quantitative experiments to show that crystalline ovalbumin is a definite hydrate. The above-mentioned observations would be then comprehensible if, due to slight autolytic changes, the protein had lost some NH_2 groupings, and thus at least some of its power of salt formation with acids, yet retained its ability to form a definite hydrate. The globuliths, on this view, would be egg-hydrate, but not potentially crystalline ovalbumin.

In the light of the above discussion it is of interest to consider the conditions under which Hofmeister first obtained crystals. He slowly concentrated a half-saturated solution of ammonium sulphate containing albumin. Now, such a solution is naturally acid, due to the fact that $(NH_4)_2SO_4$ is a salt formed by the union of a very strong acid with only a moderately strong base. Furthermore, on slow evaporation in open vessels some of this free NH_4OH will be lost, and the solution will become more acid until such a point is reached at which the hydrolysis will become negligible. It would thus be conceivable that at one stage of evaporation globuliths would appear, and, at a later stage, when the solution had become more acid, crystals. The globuliths might then take up more acid and crystallise, since the phases differ in degree of their constituents and not in kind, and the disperse phase is more or less permeable to electrolytes in the continuous one.

In any case, both crystals and globuliths are precipitated again from a solution of $(NH_4)_2SO_4$, which is much less concentrated than that required for ordinary amorphous material. The concentration of $(NH_4)_2SO_4$ is usually slightly more than quarter saturation, while amorphous albumin in equal concentration requires considerably more than half saturation. This fact in itself, to my mind, is a very good reason for believing that both crystals and globuliths are hydrates of ovalbumin. The *rôle* of $(NH_4)_2SO_4$ would thus be to remove the solvate water associated with the albumin molecular aggregates which kept them in solution, but to leave the hydrate water still attached. If the hydrate were not formed, then the concentration would have to be considerably increased in order to control this excess of free-water molecules, and an amorphous precipitate would result.

Discussion of Results.

From the experiments described in the preceding sections we have seen that there is a variation of the specific rotation of ovalbumin, depending upon the hydrogen ion concentration of the solution. The addition of acid to a solution at its isoelectric point causes an increase of its rotatory power, which remains constant. The addition of alkali to a similar solution causes a prompt fall in rotatory power, which slowly rises to the original value. Further addition of alkali has no effect. These changes are reversible from either side of the isoelectric point. If, however, the albumin sample be kept at the isoelectric point or thereabouts, without adding either acid or alkali, it is possible to obtain a constant specific rotation for successive recrystallisations.

The maximum experimental errors are small. The polarimeter readings are accurate to 0·01 of a degree, and since rotations of ten degrees or more have been observed, the error from this determination would not be greater than 0·02 per cent. The error in the estimation of the dried coagulum from variations observed might amount to 2·5 mgrm. in 0·5 grm., or a maximum error of 0·5 per cent. A fluctuation of 0·25 of a degree in the specific rotation value would be the maximum experimental error permissible. It will be found that the results agree more closely than this as a rule. The variations in specific rotation produced by variations of P_H, as shown in Table VII, for example, amount to several degrees. There seem to be two possible explanations for this phenomenon. As Fischer has shown, the salts of amino acids have different optical rotations from the free acids themselves. Now the isoelectric point must be taken as the natural point of neutrality of the protein molecule; that is, the point at which the dissociation constants of the protein as an acid and as a base balance. By the addition of acid or alkali, salts would be formed on amino acid groupings, and the measure of salt formation would be the governing factor in change of optical rotation. This is certainly not so on the alkaline side, for beyond the first drop in rotation, further addition of alkali has no obvious effect. Likewise the addition of acid can change the rotation once, and once only, apparently.

The second explanation, as suggested by Robertson, that a tautomeric equilibrium exists in certain amide groupings is most forcibly emphasised by the slow change in optical rotation in alkaline solution, and, further, by the reversibility of the phenomenon generally from either side of the isoelectric point. That such a tautomerism is probable from other reasons has been very fully shown by Robertson, in supporting his theory of protein ionisation. Furthermore, such an equilibrium would be governed by the hydrogen ion concentration, and not by the quantity of acid molecules present. The slight changes in the acidity of the medium might thus account for the fluctuations of the specific rotations observed and recorded in the literature.

Crystalline Serum Albumin.

Crystalline serum albumin was first prepared by Gürber in 1894 by the direct application of Hofmeister's method to horse serum. Merely from the appearance of the crystals, Gürber concluded that three albumins occur in serum, all crystallisable. Michel, in 1896, introduced a slight modification in the technique, and determined the coagulation temperature and specific rotation of the crystalline product. Hopkins and Pinkus (1898) mention the fact that it is possible to obtain crystals from serum by the acid method, but the time required is one to several days. Krieger (1899) shortly afterwards suggested the use of H_2SO_4 in place of acetic acid without any quantitative data. In his paper on pure ovalbumin, Hopkins mentions that, in some attempts to purify serum albumin in like fashion, more difficulty was experienced in arriving at a homogeneous product by reason of pigment contamination. The last study of serum albumin was carried out by Hardy and Gardiner, and only published in abstract form. By means of absolute alcohol and anhydrous ether as extraction solvents and dehydrating agents at −4° C., a snow-white protein was obtained, which could be readily dissolved and crystallised. This mode of preparation serves to remove all traces of fatty material present in the original precipitate from serum, and has been used in part of the experimental work. The claim is made by these authors, unfortunately on very little published experimental evidence, that the albumin in serum exists as a complex of albumin, cholesterol esters and two pigments. This point is discussed later in the light of my experimental findings.

The various values recorded in the literature for the specific rotation of serum albumin are given in Table IX.

Table IX.

Observer.	Material.	Specific rotation.
Frédéricq (1880)	Amorphous	° −56·07 to −58·41
Starke (1881)	,,	−60·05
Sebelien (1885)	,,	−60·1 to −62·6
Michel (1896)	Crystalline	−61·0
Maximovitsch (1901)	,,	−47·47

Preparation of Material.

Two methods of preparing albumin have been followed with a view to contrasting the products obtained, the alcohol-ether method of Hardy and Gardiner, and a procedure evolved from the general principles of the Hopkins-Pinkus method. The blood was drawn from the right jugular vein of a normal horse and collected in sterile winchesters, where it was allowed to clot spontaneously. The serum was siphoned off after 2 or 3 days. With one preparation the blood was first defibrinated, and then centrifuged free from corpuscles.

Method 1.—The serum obtained was transported to cold storage chambers, where it was poured into three times its volume of 95 per cent. alcohol at a temperature of −4° C. The proteins thus precipitated were allowed to stand for 24 hours, when filtration was commenced through folded filters. The bulky cream coloured precipitate was washed at −4° C. as follows: (1) three times with 95 per cent. alcohol; (2) twice with absolute alcohol;

(3) twice with absolute ether. The precipitate was next transferred to soxhlet thimbles, placed in a desiccator, and transported to the soxhlet apparatus. In this it was now extracted with absolute ether for 15 hours, after which there was no further loss in weight. The thimbles were quickly removed to a desiccator containing H_2SO_4 and thoroughly dried *in vacuo.* On cooling, cholesterol esters crystallise out from the ether used in extraction. The latter apparently extracts some pigment as well, for it becomes coloured a bright yellow. The product obtained by this procedure is a very fine snow-white powder, which can be kept indefinitely if thoroughly dried. It is necessary to keep the precipitate well protected from moist air when in contact with either alcohol or ether at a temperature above 0° C. The precipitate will otherwise turn into a brown, brittle mass on drying, which is insoluble in water. From 3250 c.c. of serum, 240 grm. of protein were obtained, representing approximately 7·4 per cent:—

Analysis of Material.

The white powder in dry form, or as a concentrated solution from which the protein had been removed by means of dialysed iron, was examined for the following substances by the tests mentioned below with negative results in every case.

(1) Cholesterol by Salkowski and Libermann-Burchard.
(2) Lipoid by organic phosphorus, choline and sulphuric acid.
(3) Fat by acrolein.
(4) Protein hydrolytic products by biuret.
(5) Glucose by Benedict's picric acid.

The powder was analysed for total non-volatile solids and for its ash-content by heating to constant weight in a platinum crucible and igniting at a low temperature. The average for three determinations of loss in weight, probably representing volatilisation of traces of alcohol and ether, was 12·36 per cent. and of ash 1·34 per cent. on the dry weight. The samples of powder remained soluble in water in spite of having been subjected to a temperature of 112° for 8 hours. This was no doubt due to the thorough dehydration.

By reason of the statement made by Hardy and Gardiner that the product obtained by them from horse serum after a somewhat similar procedure showed the normal alkalinity of serum, which, they remark, is not due therefore simply to alkaline carbonates, it was deemed important to determine the alkalinity of the powder in solution. A solution of 13·9 per cent. strength was placed in a hydrogen electrode vessel of the Barendrecht type, installed in a constant temperature chamber at 18°, connected up with a calomel electrode containing saturated KCl solution and the usual electrical appliances for the electrometric determination of hydrogen ion concentration. The Walpole medical research potentiometer was used with a Weston standard cell as standard. The E.M.F. observed was 689·5 millivolts which represents a hydrogen ion concentration of $2{\cdot}45 \times 10^{-8}$ or P_H 7·61. The solution was next diluted with an equal volume of water and the difference of potential again determined. The result was the same as before.

As the ash-content of the protein powder is quite appreciable, and as is evident from the experiments recorded below this ash is made up, at least in part, of fixed alkali, the alkalinity of the solution I would attribute to the inorganic constituents rather than to the weakly basic protein.

In the following experiments on the albumin obtained by the above method the determinations of P_H were made by the potentiometric method as used for the alkalinity determination.

Experiment 1.—A 20 per cent. solution was prepared in distilled water. It may be noted here that in spite of its content of globulin the protein powder is entirely soluble in distilled water. It would thus appear that by this method of preparation the eu-globulin is so changed that it is now quite soluble in distilled water. To the mixed protein solution was added a saturated solution of Kahlbaum's purest $(NH_4)_2SO_4$ in equal volume. As is the case with ovalbumin preparations a distinct odour of ammonia is perceptible, indicating the presence of fixed alkali. The P_H of the medium was 5·84. The globulin precipitate was allowed to settle for 24 hours. It was then filtered off and a portion of the filtrate made acid with acetic acid (10 per cent.) to the point of equilibrium found desirable in the crystallisation of ovalbumin, *i.e.* a slight permanent precipitate. No crystals were formed and the solution had a P_H of 4·16. Another portion of the filtrate was similarly treated with acid but to a less degree. Crystals appeared in 12 hours. The P_H of the mother liquor was 4·30. The remaining filtrate was treated with acid to a point when the first faint turbidity was visible. Crystals in abundance were deposited inside of an hour. The P_H of the mother liquor was 4·54. The angles of the crystals were sharp and the crystals themselves large and single, resembling small hippuric acid crystals very closely. They were filtered off and dissolved in distilled water. There was absolutely no trace of residue remaining in the fluid. The solution was coloured a yellowish-brown. An attempt to recrystallise this preparation by addition of saturated $(NH_4)_2SO_4$ solution, added to the point of the first permanent turbidity, produced not a single crystal but only a bulky amorphous precipitate in about 30 minutes.

Experiment 2.—A 15 per cent. solution was prepared and the globulin removed as previously. Acetic acid was added until the P_H was adjusted to 4·8. Crystals began to form in 2 minutes and by 5 minutes there was a heavy precipitate, consisting entirely of crystalline material. The amount of albumin which was obtained in crystalline form by this procedure was estimated at 70·4 per cent. The crystals were redissolved in water and $(NH_4)_2SO_4$ solution added with great care at a very slow rate to a point somewhat short of a permanent precipitate. On standing, the solution deposited crystals in about an hour, and the yield was increased by slow addition of $(NH_4)_2SO_4$. The crystals were again brought into solution and the liquid still showed a slight yellow colour. The rotatory power was observed and the concentration of albumin determined by the Devoto method, as previously described. The albumin was again recrystallised and the determinations repeated. Table X summarises the results, and it is thus evident that the specific rotation is constant after the first crystallisation:—

Table X.

Crystallisation.	Protein concentration.	α_D.	$[\alpha]_D.^{18}$	α_E.	$[\alpha]_E.^{18}$
	per cent.	°	°	°	°
—	0·98	−1·13	−58·06	−1·36	−69·26
First	2·18	−2·74	−62·94	−3·41	−78·20
Second	3·36	−4·29	−62·84	−5·26	−78·32
Third	1·42	−1·78	−62·70	−2·22	−78·25
Average	—	—	−62·83	—	−78·26

Method 2.—The object of this experiment was to discover whether it was possible to obtain an albumin giving the same specific rotation, but prepared by a different method. The second method of obtaining serum albumin in a pure state was evolved from attempts to obtain crystals from serum by the exact application of the method of Hopkins for ovalbumin. This method has been used by several investigators as a means of obtaining relatively pure serum albumin for various purposes, but the procedure has never been carefully studied, and it was still an open question whether an individual albumin could be obtained from serum. Furthermore, the fundamentally important relations of serum albumin to the lipoids, cholesterol, and cholesterol esters of the blood stream seemed possible of investigation by a contrast of the serum albumin obtained directly and by the isolation method previously described.

The method was constructed on the basis of a series of preliminary experiments with both CH_3COOH and H_2SO_4 in an endeavour to determine the optimum P_H conditions. It soon became evident that any amorphous precipitate which was formed before crystallisation never became crystalline. This is in sharp contrast with the behaviour of ovalbumin. The conditions for the deposition are spread over a much wider range of concentration both of hydrogen ions and of ammonium sulphate. Crystals, moreover, will continue to form as very long narrow needles for several weeks after their first appearance.

When the first crop of crystals has been obtained, centrifuged off and placed in distilled water, there is always a considerable residue, although no amorphous material can be seen amongst the crystals. The nature of this residue has been investigated and is discussed in a later section. In order to discover whether the insoluble residue remaining after the first crystallisation could be eliminated by previous washing of the serum with ether, this experiment was tried on a sample of serum with complete success. The crystals formed with greater ease, probably due to the lowering of the surface tension at the interfaces of growing crystal and mother liquor. The angles of the crystals were very sharp and the size was uniform; furthermore, the crystals dissolved rapidly in distilled water, leaving only a minute trace of insoluble matter.

The procedure which I have found successful for obtaining a large uniform yield of crystals and for recrystallisation is as follows.

The serum is washed with an equal volume of ether in several small quantities by the use of a separating funnel. Serum globulin is removed by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The precipitate is filtered off after 4 or 5 hours. The orange-yellow filtrate is slowly acidified by means of acetic acid (10 per cent.) or sulphuric acid, N/3, to the point of the first trace of turbidity, the solution being constantly stirred. This point corresponds to a P_H value of 6·0, and a pigment change from red to yellow somewhat precedes it. The cloudiness will gradually become more dense as further masses of crystals separate. After two hours the acidity is further increased by the addition of about half as much acid as previously added. Crystals will be found to form with much greater ease once their deposition has been induced at a low hydrion concentration. After another two hours a third portion of acid is added, equal to the second. This should establish the P_H of 4·9 to 5·1, below which it is not advisable to go. The yield of crystalline material may be further increased by addition of more $(NH_4)_2SO_4$ solution, a few cubic centimetres at a time (1 c.c. per 100 c.c. of fluid) until precipitation ceases. The crystals so separated go into solution practically without residue, but the solution is strongly pigmented. Crystallisation can be repeated without much loss of material if care is used in adding the $(NH_4)_2SO_4$ with sufficient slowness. This operation is more difficult than the corresponding one with ovalbumin in that to produce a permanent precipitate would be quite fatal to crystal formation. $(NH_4)_2SO_4$ as a saturated solution must be added to a point when the precipitate, which first forms temporarily, redissolves slowly. From this on it is safest to add only a few cubic centimetres every 20 or 30 minutes (1 c.c. per 100 c.c. fluid). By this method I have obtained 82 per cent. of the total albumin in crystalline form. I have crystallised serum which had remained for six months at −4° C., so that the factor of staleness would not appear to affect the crystallisation of serum albumin to the extent that it does that of ovalbumin.

Again, I would emphasise that, as in the case of ovalbumin, we are obviously treating an equilibrium reaction which is difficult to force to completion. The albumin remaining in solution need not of necessity be looked upon as non-crystallisable or as essentially different from the crystalline. It may be that, if it were possible to control physically all the constituents of the reaction, and to alter these as they were changed by the deposition of crystals, then the albumin obtained in crystalline form would amount to nearly 100 per cent. The physical difficulties, however, become greater as the concentration of albumin in solution becomes less. The fraction of total albumin crystallised, viz., 82 per cent., is somewhat higher by this method than that obtained by the first method, viz., 70 per cent. In the first method, however, the concentration of albumin was much lower, owing to the small amount of material available. It was thus more difficult to control the distribution of water between remaining albumin, $(NH_4)_2SO_4$, and free acid, while maintaining the latter at its optimum concentration.

By the above method, it has been possible to carry through a series of crystallisations with determinations of rotatory power. Table XI summarises the results obtained:—

Table XI.

Crystallisation.	Temperature.	Concentration.	$[\alpha]_D$.	α_E.	$[\alpha]_E$.
	° C.	per cent.	°	°	°
First	20	5·00	—	−3·73	−74·78
Second	17	5·41	—	−4·09	−75·71
Third	15	6·08	−62·98	−4·77	−78·60
Fourth	20	2·34	−62·70	−3·08	−78·24
Average	—	—	−62·84	—	−78·42

The third and fourth values are practically identical when the limits of accuracy of the determination of the albumin are considered. It is, moreover, of great interest to note that the value coincides with that obtained by the alcohol-ether method, as recorded in Table X.

Discussion of Results.

There has been much discussion for years past as to the possible chemical alliance of proteins with fats or lipoids in the blood. This is particularly true of eu-globulin, and Hardy and Gardiner have briefly indicated that it is possible that serum albumin is chemically bound to cholesterol esters and pigments in serum. Now, it is necessary to recognise that, in determining the value for specific rotation, we really have only allied rotatory power with coagulable material. That is to say, that identical weights of coagulated material in undenatured form from the two preparations, if made into solutions of identical concentration, would have an identical rotation. If cholesterol were present in chemical union with one albumin, then it must of necessity have an inappreciable effect upon the total observed rotation, and be completely volatilised during the drying operation. By reason of the fact that cholesterol is optically lævo-rotatory, these circumstances are, to say the least, improbable. One other possibility must be mentioned. The cholesterol, or other fatty material with which the albumin was chemically associated in serum, might be split off in the act of crystallisation. The "milieu" for such a chemically conceived separation is, indeed, nothing more vigorous than a concentrated $(NH_4)_2SO_4$ solution and very dilute acetic acid at a temperature of 20°. At the same time, proteins are extremely labile molecular structures. There is, furthermore, one piece of concrete evidence. On first crystallisation direct from serum, there is an insoluble residue of a fatty nature. The deposition of this residue is completely inhibited by previous ether extraction. The crystals themselves are most probably a pure protein, and of the same nature as found for ovalbumin, although differing in their chemical structure of amino acids, as indicated by the difference in specific rotation.

The solutions used for the determinations of specific rotation recorded were never entirely free from pigment. The solutions from the first method were much clearer than those from the second. In the latter, the successive recrystallisations diminished the pigmentation of the solution but little. I have tried many methods of removing pigment by adsorbents without success, *e.g.*, kieselguhr, charcoal and freshly precipitated barium sulphate. If the crystals are produced very slowly, the first crop formed is always much more coloured than subsequent ones. The amount of pigment, however, must be minute, since concentrations of over 5 per cent. of albumin can be examined in 200 mm. polarimeter tubes with ease. The work of Palmer and Eckles (1914) tends to show that the pigments are carotin and xanthophyll of plant tissues. But since carotin, which predominates greatly in horse serum, has such an intense colouring power, the amount in the solution used optically must be very small—less than 1 mgrm. in 100 c.c.

The true relationship between the fatty material or pigment and the protein in natural serum is best understood, to my mind, by the concept that in such complex compounds as the proteins there is no sharp distinction between the so-called physical adsorption and chemical union of associated substances. As Langmuir has pointed out from inorganic studies adsorption is fundamentally chemical.

The Insoluble Residue.

I have examined the residues which remained after the first crystallisation of several lots of albumin from serum. It is very obvious that they differ markedly in texture. Occasionally they can be collected with difficulty on account of being an oil. As such it is impossible to centrifuge and difficult to filter. When separated it is found to be entirely and readily soluble in ether, except for a little contaminating protein. This solution shows a very heavy precipitate on adding acetone but an oil is deposited on slow concentration. At other times the residue is a solid which can be readily centrifuged off and washed free from pigment. I have dissolved this in ether and hot alcohol. On cooling the solution in alcohol deposits, needles of the appearance of cholesterol esters and the solutions give a very strong cholesterol reaction.

Since no amorphous material is ever visible under the highest power of the compound microscope when the first crystalline product is examined, yet a very marked residue remains on attempting resolution, I examined the crystals while they were going into solution under the microscope. It then became clear why the albumin dissolved so slowly. As the highly refractive protein interior of the crystal disappeared there still remained the outline of the crystal. Soon the field became covered with these shells of the former crystals without any remaining protein. There was still no amorphous material. On agitating the slide, however, the crystal outlines rounded into fat-like globules. It would thus appear that each crystal had had a thin covering of fatty material deposited about it. This would naturally interfere with the process of crystallisation and make the crystal sizes vary. It is noticeable that large crystals are first formed which are four or five times the mass of smaller ones appearing later. Furthermore the presence of this fat-like material alters the shape of the crystal, obliterating angles and inhibiting rosette formation. When the serum is ether-washed previously to crystallisation, the crystals show sharp angles, are of uniform size and more quickly deposited.

The above observation shows how the nature of the medium may affect the structure of the crystals and is of interest in the consideration of all the work which has been done in the endeavour to link up crystalline form of proteins with plant or animal species from material which has been crystallised but once from its natural medium. It is also interesting in showing the possible physical associations between lipoid and protein material in protoplasm and the difficulty of removing the one constituent without affecting others.

SUMMARY AND CONCLUSIONS.

Crystalline Ovalbumin.

The specific rotation of crystalline hen ovalbumin is found to be $-30{\cdot}81^\circ$ for $[\alpha]_D{}^{15}$ and $-37{\cdot}53^\circ$ for $[\alpha]_E{}^{15}$. The value for $[\alpha]$ is constant within the limits of experimental error after the second crystallisation if recrystallisation be carried out about the isoelectric point (P_H 4·9–5·1).

At a lower hydrion concentration (P_H 5·3–5·4) a constant lower specific rotation is obtained, $[\alpha]_D{}^{15} = -30{\cdot}14^\circ$ and $[\alpha]_E{}^{15} = -36{\cdot}80^\circ$. The results were identical by two different methods of analysis.

The optical rotation of an albumin solution at its isoelectric point remains constant. If it be made slightly acid a prompt rise in rotatory power is observed to a new constant level. If the solution be made alkaline to P_H 4·9 a prompt fall in rotatory power ensues which very slowly rises to the previous value. This phenomenon can be brought about from either side of the isoelectric point and is reversible. The variations in optical rotation are explained on the basis of a tautomeric equilibrium of the lactam-lactim type.

Experiments are recorded which tend to show that globulith formation is no indication of the power of a protein to crystallise, but probably indicates hydrate formation.

Crystalline Serum Albumin.

Two methods have been used for the preparation of pure serum albumin (horse). The first method involves the precipitation and complete dehydration of the mixed proteins of serum at a temperature of -4° C., by means of absolute alcohol and ether, removal of fatty substances by Soxhlet extraction, and crystallisation of albumin from aqueous ammonium sulphate solution; 70·4 per cent. of the total albumin was obtained in crystalline form. After the first crystallisation the product possesses a constant rotatory power, $[\alpha]_D{}^{18} = -62{\cdot}8^\circ$, $[\alpha]_E{}^{18} = -78{\cdot}3^\circ$.

The second method involves the extraction of the serum by ether and the crystallisation of the albumin by ammonium sulphate and acid at P_H 6·0 to 5·0; 82 per cent. of the total albumin is thus obtained crystalline. After the third crystallisation the specific rotation is constant, $[\alpha]_D{}^{18} = -62·8°$, $[\alpha]_E{}^{18} = -78·4°$.

The insoluble residue from the first crystallisation from natural serum is shown to be a deposit of fatty material around the crystal surface. Its nature varies with different preparations, sometimes phosphatides predominate: sometimes cholesterol esters. The relation of fatty material and protein in serum is discussed.

In conclusion I wish to express my sincere thanks to Prof. F. Gowland Hopkins for his valuable advise and criticism during the course of this research.

REFERENCES.

Alexander, A. C., 'J. Exper. Med.,' vol. 1, p. 304 (1896).
Bradford, S. C., 'Biochem. J.,' vol. 14, p. 91 (1920).
Chick, H., and Martin, C. J., 'Biochem. J.,' vol. 7, p. 380 (1913).
Clark, W. M., and Lubs, H. A., 'J. Bacter.,' vol. 2, pp. 1 and 109 (1917).
Gürber, A., 'Sitzungs. Würzburger Phys.-med. Ges.,' p. 143 (1894).
Hardy, W. B., and Gardiner, Mrs. S., 'Proc. Physiol. Soc., J. Physiol.,' vol. 40, p. 68 (1910).
Hopkins, F. G., 'J. Physiol.,' vol. 25, p. 306 (1900).
Hopkins, F. G., and Pinkus, S. N., 'J. Physiol.,' vol. 23, p. 130 (1898).
Krieger, H. Th., 'Inaug.-Dissertation,' Strassburg, from Maly (1899).
Michel, A., 'Verhand. Würzburger Phys.-med. Ges.,' p. 29, No. 3 (1896).
Osborne, T. B., 'J. Amer. Chem. Soc.,' vol. 21, p. 477 (1899).
Osborne, T. B., and Campbell, G. F., 'J. Amer. Chem. Soc.,' vol. 22, p. 422 (1900).
Oswald, A., 'Z. physiol. Chem.,' vol. 95, p. 102 (1915).
Palmer, L. S., and Eckles, C. H., 'J. Biol. Chem.,' vol. 17, p. 223 (1914).
Pauli, W., Samec, M., and Strausz, E., 'Biochem. Z.,' vol. 59, p. 470 (1914).
Robertson, T. B., 'Die physikalische Chemie der Proteine,' Dresden (1912).
Sörensen, S. P. L., 'Erg. Physiol.,' vol. 12, p. 393 (1912).
Sörensen, S. P. L., et al., 'Compt. Rend. Lab. Carlsberg,' vol. 12 (1917).
Willcock, E. G., 'J. Physiol.,' vol. 37, p. 27 (1908).